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Rebuilding immunity in cancer patients

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Abstract

Rebuilding and maintaining immunity is paramount to the success of cancer immunotherapy and hematopoietic stem cell transplantation. If immune surveillance indeed can protect from cancer, the very manifestation of malignancy means that the disease has prevailed over immunity. Yet, often, tumor-specific T cells can be found in cancer patients irrespective of vaccination. Interestingly, patients suffering from malignancy often harbor unexpectedly high levels of immature CD14⁺HLA-DR⁻ monocytes, although the abundance of CD4⁺ cells, CD8⁺ cells and CD4⁺CD25^{high} cells may be normal. It is plausible that in cancer such cells suppress T cell function, analogous to CD14⁺HLA-DR⁻ cells in sepsis and major trauma, in addition to their likely failure to re-present tumor-associated antigens once dendritic cells have initiated the T cell response. Recent evidence indicates that tumor-borne adenosine, lactate and hypoxia in the tumor environment may modulate tumor-specific immunity to a significant extent, but their effects on myeloid cell function is unclear. Thus, understanding and controlling these factors may appreciably impact the success of rebuilding and maintaining immunity in cancer patients.

Cynics would say that there are more literature reviews and opinion papers on cancer immunotherapy than there are patients cured. Indeed, since the time of Coley's toxins in the early 20th century (1) the efforts to engage immunity to fight cancer have included the use of adjuvants (*e.g.*, *Bacille Calmtte-Guerin* or purified bacterial products; (2), monoclonal antibodies (3), lymphokine activated killer cells (4), tumor infiltrating lymphocytes (5), interleukin 2 (6-8) and other cytokines (9), T cells (10) and dendritic cells (11), to name just a few. The general objective has been to stimulate the immune system to recognize and eliminate tumor cells. Yet very few of these modalities, besides a limited number of monoclonal antibodies, have become a standard complement to the treatment of malignant diseases. This review examines evidence that patients suffering from malignant diseases harbor not only ineffective T cells, but also dysfunctional myeloid cells that contribute to the failure of immunity to respond to vaccination in a clinically effective manner. Tumor-borne adenosine, lactate and hypoxia appear to play a role in suppression of myeloid function so we consider approaches that might control these factors and aid in the rebuilding of immunity.

Recent efforts in cancer immunotherapy have successfully defined select molecular targets (epitopes) associated with cancer cells (12); these efforts resulted in the increased efficacy of raising tumor-specific immune effector cells (10,13). Melanoma has been a particular subject of interest, so numerous tumor-associated epitopes have been characterized on this tumor (14). Many epitopes have been tested in clinical trials as have been other approaches to

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vaccination with tumor-associated antigens: autologous (15,16) and allogeneic whole-cell cancer vaccines (17) that can be genetically modified to secrete cytokines (18,19), cancer cell lysates (20), transcripts isolated from cancer cells (21), heat shock proteins (22), viral constructs (23), defined tissue-associated antigenic proteins (24) and others.

Advances in defining tumor-associated antigens have been followed by more efficacious modes of vaccination utilizing novel adjuvants such as imiquimod (25), monophosphoryl lipid and derivatives (26), and antigen-presenting cells (24), particularly dendritic cells (11). These innovations have been tested in numerous clinical trials that, in general, successfully raised tumor-specific T cells; nonetheless, clinical responses have been marginal (15); *cf.* the compendium of dendritic cell trials at <http://www.mmri.mater.org.au/>.

Cancer patients harbor tumor-specific T cells irrespective of vaccination

Interestingly, tumor-specific T cells can be detected in patients' circulation even before vaccination. Hellström and colleagues observed this phenomenon, sometimes named "The Hellström Paradox," some forty years ago (27,28). The paradox implies that the immune system registers tumor cells as distinct antigenic entities and raises T cells that recognize them—yet the tumor persists. In other words, when the tumor becomes clinically manifest, it has long won over immunity (29,30). This means that therapeutic vaccination against tumors takes place within a milieu of hyporesponsive immunity. While regulatory T cells (31) and suppressive cytokines (32-34) have been recognized as factors in suppression of tumor-specific T cells, their role in the natural history of disease is unclear. Has the tumor "won" because regulatory T cells were present at tumor initiation, or are these cells manifest in the cancer patient because the disease skewed T cell development towards regulatory T cells? Successful resolution of these questions is critical for the question why therapeutic tumor vaccination works only marginally.

The Hellström Paradox demands answers, *inter alia*, to the mutually non-exclusive questions whether the spontaneously raised immune effector cells recognize therapeutically relevant cellular targets within the heterogeneous tumor cell population and whether the tumor renders effector cells ineffective. Recent advances in the understanding of mechanisms of tumor evasion from immunity (35) and of existence and role of cancer stem cells (36) shed some light on the possible origins of the Hellström paradox.

Discovery of cancer stem cells requires a reexamination of tumor immunotherapy

When fluorescence of murine bone marrow stained with the fluorogenic dye Hoechst 33342 was measured concomitantly in the blue and the red parts of the spectrum, a small number of cells fluoresced weakly and were thus recorded in the scatter plots on the side of more fluorescent cells; an analysis of surface markers identified these "side populations" as hematopoietic stem cells (37). Cancer tissues and cultured cancer cells also contain side populations (38); this observation is in line with the hypothesis that cancer is a stem cell disease, *i.e.*, that cancer is (re)generated by a small number of slowly proliferating cells (39). Cells of the side population are characterized by effective detoxification mechanisms (hence the weak fluorescence due to less retained dyestuff compared to the more differentiated cells; (38)).

Implications of the hypothesis of cancer as a stem cell disease are numerous: Is the therapeutic targeting of the (pheno)typical cancer cells sufficient for eradication of cancer? Do we need to target cancer stem cells specifically? Is success of therapy predicted by the effects on cancer stem cells, rather than on the malignant mass? Is cancer resistance to chemotherapy due to cancer "stemness"? Is minimal residual disease comprised of stem cells? Coupled with the fact

that no specific means is yet available to deal with cancer stem cells, the answers to these questions are likely to redefine our concepts of cancer and its treatment, including immunotherapy.

An important corollary to the cancer stem cell hypothesis is that cancer stem cells may exhibit antigenic signatures distinct from pertinent normal stem cells. This hypothesis has not been tested yet in clinical trials, but—if proven—may greatly impact the approaches to cancer vaccines and cancer immunotherapy. This may be particularly so in the application of whole cancer-cell vaccines that exhibit a broad range of tumor antigens, possibly including antigens associated with cancer stem cells.

Aneuploidy could constitute another factor with a role in antigenic signature of cancer cells. Aneuploidy has been implicated in cancer biology for a long time, but its precise role remains controversial (40). In fact, the temporal and causal relationship of aneuploidy and mutations of defined oncogenes in cancerogenesis is still unclear (41). Whatever that relationship, transcriptome analysis of cancer cells reveals a systemic gene deregulation (42) with up till now unpredictable consequences for the definition and stability of antigenic signature of cancer. Like such signature of cancer stem cells, the role of aneuploidy in cancer immunology remains widely open to scrutiny. Thus, it may well be that the current cancer vaccines do not address the likely distinct antigenic signatures of cancer stem cells and cells of varying degrees of aneuploidy.

Patients suffering from malignancy often harbor unexpectedly high levels of immature HLA-DR⁻ monocytes

The uncertain role of cancer stem cells and tumor cell aneuploidy provide the rationale for the use of whole cancer cells as vaccines (15). The justification is that such vaccines can present a comprehensive array of relevant antigens, including those putatively associated with cancer stem cells and aneuploid cancer cells. Because only dendritic cells are endowed with the ability to activate naïve T cells (43), we are now studying the role of *ex vivo* matured autologous dendritic cells in enhancing the immune and therapeutic effects of an allogeneic prostate cancer whole-cell vaccine (17) in a phase 2 clinical trial.

To validate the procedure for *ex vivo* preparation of mature myeloid dendritic cells from monocytes isolated from prostate cancer patients, recently we analyzed isolated peripheral blood mononuclear cells of prostate cancer patients. Although these patients exhibited a normal abundance of CD4⁺ cells, CD8⁺ cells and regulatory CD4⁺CD127⁻ T cells (44), their blood contained high levels of CD14⁺HLA-DR⁻ cells relative to healthy controls. To determine the ability of these phenotypically immature monocytes to differentiate into mature dendritic cells, we isolated them by CD14-specific immunomagnetic adsorption and matured into dendritic cells. Dendritic cells prepared from monocytes of prostate cancer patients expressed lower levels of the maturation marker CD83 and chemokine receptor CCR7 than normal dendritic cells indicating either that CD14⁺HLA-DR⁻ cells cannot mature into dendritic cells or that they inhibit maturation of those that can.

The role of HLA Class II⁻ monocytes is well documented in sepsis and major surgical trauma

The occurrence and role of immature monocytes in cancer patients has been considered before, particularly with respect to the impaired development of these cells into dendritic cells and the role of cytokines such as IL-10, vascular endothelial growth factor (VEGF), transforming growth factor- β (TGF- β), etc. (45). In the late 1970s and early 1980s, several observations documented the reduced immune reactivity in the patients suffering from bladder and prostate

cancer; the cells isolated from patients' lymph nodes (46) and peripheral blood (47) inhibited the mixed leukocyte culture. It was first inferred (47) and then demonstrated (48) that the cells exerting this effect were monocytes. Such sporadic observations continued. For example, immature HLA-DR⁺ monocytes were identified in the peritoneal exudates from the ascites of ovarian cancer patients (49). These cells expressed immunosuppressive cytokines IL-10 and TGF- β , but did not express stimulatory molecules IL-12 and tumor necrosis factor- α (TNF- α) or antigen-presenting and costimulatory molecules (*e.g.*, HLA-DR, CD80, CD86). In addition, HLA-DR⁺ monocytes inhibited phytohemagglutinin-stimulated proliferation of autologous T cells (49). Persons suffering from malignant melanoma harbor peripheral blood monocytes that express only low levels of HLA-DR, HLA-DQ, HLA-DP and CD86 molecules in comparison with healthy controls (50). Interestingly, expression of these molecules diminished with disease progression indicating the nexus between the status of myeloid cells and disease.

The significance of HLA Class II⁺ cells in natural history of cancer can be inferred from the role of such cells in sepsis, systemic inflammatory response syndrome (51,52) and major surgical trauma (53) where they play a significant role in immunosuppression. For example, reduced HLA-DR expression on CD14⁺ cells augurs a poorer outcome after surgery, transplantation and sepsis (52); the underlying mechanisms of the associated immunosuppression is not clear, but diminished monocyte capacity for expression of TNF- α , IL-1 α , IL-1 β , IL-6 and IL-12, associated with increased expression of IL-10 may be involved. Interestingly, the presence of interferon- γ (IFN- γ ; *ref.* 54) or granulocyte-macrophage colony-stimulating factor (55,56) in culture media can improve the immune function of these defective human monocytes and may thus counteract the effects of sepsis on immunity. Similarly, HLA-DR expression on CD14⁺ monocytes and serum IL-10 levels in pediatric lung transplant patients affect clinical outcome; the patients whose HLA-DR expression did not decline or recovered within two weeks after transplantation developed neither pneumonia nor other infection nor did they reject the tumor (53).

In the absence of a direct model for the function of HLA class II⁺ monocytes in cancer, it is useful to consider endotoxin-tolerant monocytes; the cells become tolerant following acute exposure to endotoxin when they cannot present antigens and initiate antigen-specific T-cell responses. Such monocytes express little HLA class II and costimulatory CD86 molecules and cannot stimulate recall-antigen-dependent T-cell proliferation and IFN- γ release (57). These effects can be countered by neutralizing IL-10 antibodies during monocyte priming with endotoxin (57). Thus, low expression of HLA class II molecules renders monocytes unable to present antigens and stimulate antigen-specific T cells.

HLA Class II⁺ monocytes could suppress cancer-specific immunity by multiple mechanisms

In analogy to sepsis and trauma, it is plausible that HLA class II⁺ monocytes directly inhibit the action of tumor-specific T cells (45). However, most cancers are chronic diseases in comparison to the acute states of sepsis and trauma. It is therefore likely that monocyte-mediated immunosuppression in cancer is exerted through additional mechanisms that require more time to develop. For example, HLA class II⁺ monocytes fail to develop into functional dendritic cells² (58) resulting in a deficit of antigen-presenting cells uniquely able to stimulate naïve T cells (59). This notion is supported by an analysis of dendritic cells derived from monocytes of myeloma patients that revealed that the cells express less membrane-bound CD1a, CD40, CD80, and HLA-DR molecules and activate less efficiently the allogeneic and autologous T cells (58). Incubation with neutralizing IL-6-specific antibodies and a p38 inhibitor restored the phenotype and function of these dendritic cells indicating, importantly, that the defects are reversible (58). Furthermore, HLA class II⁺ monocytes are unlikely to

develop into fully functional macrophages, in analogy to their less efficient development into dendritic cells. The lack of functional macrophages will deprive immunity of the cells that infiltrate the site of primary T-cell-mediated killing, digest the resulting debris and re-present antigens from the killed cells (60). This could result in the failure to maintain the duration and amplitude of T cell attack on tumor cells. It may be worthwhile to quantify this role of tumor antigen re-presentation in clinical efficacy of therapeutic vaccination against tumors.

Free adenosine in the tumor environment may significantly modulate immunity

While regulatory T cells and cytokines received much attention for their role in suppression of tumor-specific T cell immunity, there is increasing evidence for the similar role of small molecules such as oxygen, adenosine and lactate. For example, inflamed non-cancerous tissues generate free adenosine under hypoxic conditions (*i.e.*, conditions standard in cancer and inflammation; ref. 61). Free adenosine suppresses T cells by binding to their surface A2 receptors (62-64). While in normal tissues adenosine protects from potentially autoimmune T cells, it is likely that the same mechanism protects hypoxic tumor tissues from tumor-specific T cells (65). Interestingly, caffeine is an antagonist of adenosine (66); it is tempting to speculate that the reason coffee drinkers are less prone to gastrointestinal cancers (67) stems from the caffeine function as an adenosine antagonist.

While the role of adenosine in regulating T cell responses is becoming apparent in some detail (61,68), adenosine effects in monocytes have been explored less fully (61). However, what is known is in line with the immunosuppressive effects found in T cells. Adenosine inhibits lipopolysaccharide-stimulated secretion of IL-12 (69) and TNF- α (70) and stimulates secretion of IL-10 by human monocytes (69); these effects are mediated by the A(2A) receptors. In a human monocytic cell line, IL-1 and TNF- α synergized with adenosine in suppression of IL-12 and stimulation of IL-10 expression, while IFN- γ exerted the opposite effect (71). Apparently, the underlying mechanism is an adenosine-mediated increase in intracellular cAMP in the cells incubated with IL-1 or TNF- α . The increase in cAMP levels stimulates transcription and translation of the A(2A) receptor; IFN- γ exerts the opposite effect (71). Moreover, increased cAMP levels inhibit the IL-18-stimulated expression of the adhesion molecule ICAM-1, but also of IL-12, IFN- γ and TNF- α (72). In line with other studies, an adenosine antagonist specific for the A(2A) receptor abolished the adenosine effects, but—interestingly—antagonists to A(1) or A(3) adenosine receptors synergized with adenosine. This effect is explained by the observation that activation of A(2A) receptors elevates cAMP levels, but that of A(1) or A(3) receptors reduced these levels (72). Thus, excess adenosine, such as in the microenvironment of tumors, can suppress not only the hitherto studied Th1 responses, but also the normal myeloid cells. Still, the evidence for adenosine effects in human monocytes is sparse, but so far it all points towards skewing of monocytes into the IL-10 secretion pattern. However sparse, the evidence is sufficient to warrant more detailed studies into the effects of tumor-borne adenosine on myeloid cells.

Known for the long time as a tumor companion, lactate has not been studied as a major regulator of immunity

In the 1920s, Otto Warburg recognized that generation of lactate by anaerobic metabolism is a hallmark of tumors (73,74). However, the physiological role of lactate as a major microenvironmental factor in hypoxic tissues has been studied only much more recently and its role in cancer is largely unexplored. T.K. Hunt and colleagues studied the role of hypoxia and lactate in the regulation of VEGF expression by macrophages and found that both stimulate VEGF transcription and secretion (75). Later, they demonstrated that lactate stimulates

macrophages and other cells to secrete VEGF independently of hypoxia, a known VEGF inducer (76). When they increased the levels of extracellular lactate, the levels of VEGF and TGF- β increased (76). While these phenomena may accelerate wound healing, it is noteworthy that both VEGF (34) and TGF- β (77) are immunosuppressive.

Human leukocytes, including monocytes, express monocarboxylate transporters 1, 2, and 4 (78). This feature can explain the observation that monocytes cultured in the presence of high lactate concentrations (either added to the culture medium or generated by the co-cultured multicell tumor spheroids) differentiated into defective dendritic cells akin to those found inside tumors or cultured in the presence of VEGF, IL-6, IL-10 or macrophage-colony stimulating factor (79). Under identical conditions, but with lactate levels reduced, dendritic cells reverted to control phenotype (79). Thus, modulation of lactate levels ought to be explored as a means of manipulating immunity.

Manipulating oxygen levels in tumors and immune cells may provide additional means of rebuilding immunity

To determine the effects of changes in oxygen pressure on the antigenic signature and expression patterns ovarian cancer cell lines intended for the use as therapeutic vaccines, initially we measured the expression of VEGF and intracellular hypoxia-inducible factor 1 α (HIF-1 α) as a function of oxygen pressure. Hypoxia enhanced the rate of VEGF secretion. Hyperoxia abolished proliferation, but unexpectedly boosted VEGF secretion. We compared HIF-1 α levels in normoxic cells and cells hypoxic or hyperoxic for 18 to 24 hours; HIF-1 α levels in hypoxic cells were higher than in normoxic. Interestingly, hyperoxic cells also expressed high HIF-1 α levels paralleling high VEGF secretion. Thus, ovarian carcinoma cells respond both to hypoxia and hyperoxia by elevated levels of HIF-1 α and VEGF indicating that manipulation of oxygen pressure (such as in hyperbaric oxygen therapy) can affect the release of immunosuppressive mediators by the tumor. Similarly, hypoxia enhances migration and invasiveness of ovarian cancer cells in response to lysophosphatidic acid, a compound found at elevated levels in ascitic fluid of ovarian cancer patients (80).

The effects of hypoxia and reoxygenation on human monocytes and macrophages have been studied largely for their role in ischemic injury and reperfusion. Observations of hypoxia-induced reduction of CD80 expression and stimulation of TNF- α secretion and their reversal by reoxygenation have been interpreted as reduced ability of monocytes to initiate adaptive immune responses (81). Hypoxia increased the levels of cyclooxygenase 2 in human monocytes, but paradoxically reduced the synthesis of prostaglandin E₂ and thromboxane A₂ (82). These conditions decreased phosphorylation of the p44/42 mitogen-activated protein kinase and cytosolic phospholipase A₂ and stimulated TNF- α expression suggesting that hypoxia is a major regulator of inflammatory mediators (82). These conclusions have been corroborated by a global transcriptome analysis of hypoxic monocytes that revealed “profound changes in the gene expression pattern” (83); among some 600 genes affected by hypoxia, many are known to regulate the immune functions of monocytes (*e.g.*, scavenger receptors; immunoregulatory, costimulatory, and adhesion molecules; chemokines/cytokines/chemoattractants and receptors). While such studies still cannot predict the behavior of the cell in a particular microenvironment, oxygen tension appears to exert a major effect on the regulation of immune functions in monocytes.

TNF- α induces expression of matrix metalloproteinase-9 (MMP-9) in human monocytes that allows them to move through extracellular matrix; hypoxia counters this effect by affecting posttranslational cellular trafficking of MMP-9 and its attachment to the membrane (84). Similar effects of hypoxia have been observed in human monocyte-derived dendritic cells—reduction in MMP-9 levels accompanied by reduced cell migration (85). Thus, hypoxia is

another factor that affects the immune response (61). Manipulation of tumor-associated hypoxia, together with adenosine and lactate, can provide powerful means in the efforts to rebuild patient immunity.

Oxygen levels affect stem cell mobilization into circulation

The preceding discussion indicates that modulation of tumor-borne immunosuppression by small molecules, such as adenosine, lactate and oxygen may hold promise for additional means to rebuild immunity. New opportunities for manipulation of hematopoietic stem cells and progenitors with as yet insufficiently appraised clinical potential has been afforded by the observations of mobilization of bone marrow cells by hyperbaric oxygen therapy (86). In response to a single 120-min exposure to pure oxygen at the pressure of 200 kPa (2.0 ATA), the levels of circulating CD34⁺ cells doubled. A typical twenty-day course of HBOT elevated the levels of circulating CD34⁺ cells up to eightfold relative to pretreatment levels. Studies in animals demonstrated that HBOT-mobilized CD34⁺ cells selectively localize in ischemic wounds indicating that this may be the mechanism of HBOT-induced healing of such wounds in clinical practice (86,87). Because CD34⁺ cells include not just the progenitors of vascular endothelium, but also hematopoietic stem cells and progenitors, the role of mobilizing patient's own CD34⁺ cells should be explored in regard to its role in rebuilding immunity. The effects of oxygen pressure on hematopoietic stem and progenitor cell engraftment, graft expansion, development and function of dendritic cells (both from the host and the graft), expression of tumor-associated antigens and others may provide fertile areas of basic and translational research.

Perspectives

Chemotherapy, conditioning in preparation for hematopoietic stem and progenitor transplantation, and graft-versus-host disease induce tissue damage probably analogous to that in solid organ transplantation. Consequently, many of the effects on the immune system and its rebuilding will be similar and lessons from tumor vaccination, sepsis and solid organ transplantation might be applicable. It is plausible that the failure of tumor vaccination to elicit predictable therapeutic outcomes results, at least in part, from the fact that vaccines are delivered to hyporesponsive immunity. It is intriguing to speculate whether rebuilding of full immunity in cancer patients in the absence of other treatment would result in cancer remission even without vaccination. To test this hypothesis, we must find means to rebuild suppressed immunity. In that effort, it is likely that no single treatment will succeed and that numerous treatment modalities will have to be synergistically employed.

While the literature abounds with pertinent and attractive molecular targets for reversal of immunosuppression (88), few are clinically applicable. Clinically effective neutralization of macromolecular mediators of immunosuppression (*e.g.*, VEGF, TGF- β) by monoclonal antibodies may be long in waiting, but combinations of cytokines (89,90), possibly coupled with adenosine analogues and prevention of lactate accumulation could be more promising. Effects of hyperbaric oxygen should be explored, particularly with reference to tumor biology and the possibility that oxygen treatment may unfavorably affect the natural history of disease. Finally, modern methods of transfusion medicine and clinical-grade cell manipulation are available to study effects of clinical interventions, from the relatively crude plasma exchange for removal of soluble immunosuppressive mediators (91-93), to the more sophisticated *ex vivo* cell engineering to restore cellular immune functions.

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